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Critical Roles for the Bcl-3 Oncoprotein in T Cell–Mediated Immunity, Splenic Microarchitecture, and Germinal Center Reactions

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Summary

Chromosomal translocations of *bcl-3* are associated with chronic B cell lymphocytic leukemias. Previously, we have shown that Bcl-3, a distinct member of the IκB family, may function as a positive regulator of NF-κB activity, although its physiologic roles remained unknown. To uncover these roles, we generated Bcl-3-deficient mice. Mutant mice, but not their littermate controls, succumb to T. gondii owing to failure to mount a protective T helper 1 immune response. Bcl-3-deficient mice are also impaired in germinal center reactions and T-dependent antibody responses to influenza virus. The results reveal critical roles for Bcl-3 in antigen-specific priming of T and B cells. Altered microarchitecture of secondary lymphoid organs in mutant mice, including partial loss of B cells, may underlie the immunologic defects. The implied role of Bcl-3 in maintaining B cells in wild-type mice may related to its oncogenic potential.

Introduction

bcl-3 was first identified as a gene involved in recurrent translocations in certain cases of B cell chronic lymphocytic leukemias (B-CLL) (Ohno et al., 1990). Reciprocal translocations of *bcl-3* with the immunoglobulin locus resulted in higher and possibly inappropriate expression of this gene due to the influence of a now juxtaposed immunoglobulin enhancer. Since the structure of the encoded protein was apparently unchanged, abnormal expression of Bcl-3 most likely contributed to B cell tumorigenesis, although potential mechanisms remain unknown.

Bcl-3 features a central domain with seven ankyrin repeats whose sequences are most similar to those present in IκB family proteins (Hatada et al., 1992). Like IκB proteins, Bcl-3 physically interacts with NF-κB proteins, primarily via its ankyrin domain (Wulczyn et al., 1992; Bours et al., 1993; Franzoso et al., 1993). However, unlike IκB proteins, Bcl-3 preferentially interacts with p50 or p52 homodimers (Franzoso et al., 1992, 1993; Wulczyn et al., 1992; Bours et al., 1993; Fujita et al., 1993; Naumann et al., 1993; Nolan et al., 1993). These homodimers do not readily transactivate κB-dependent reporters, because p50 and p52 are the only members of the family of NF-κB polypeptides that do not harbor known transactivation domains (for review see Baeuerle and Henkel, 1994; Siebenlist et al., 1994; Verma et al., 1995; Baldwin, 1996). Instead, p50 homodimers competitively inhibit transactivation mediated by p65-containing NF-κB complexes, and cotransfection of Bcl-3 can reverse the inhibition, apparently by removing p50 homodimers from DNA (Franzoso et al., 1992, 1993; Inoue et al., 1993). In this scenario, Bcl-3 facilitates transactivation, in contrast with the usual inhibitory functions of the other known members of the IκB family.

Bcl-3 can also function as a potent coactivator with p52 homodimers (Bours et al., 1993; Chang et al., 1995). Bcl-3 was shown to transactivate a κB-dependent reporter in the presence of p52 homodimers, apparently as a result of ternary complex formation on DNA and because of transactivation domains present in Bcl-3. In this series of experiments, exogenous expression of p50 homodimers with Bcl-3 did not lead to significant transactivation, but in another study, which differed in experimental details, this combination led to modest levels of transactivation (Fujita et al., 1993). There is evidence to suggest that the functional interaction of Bcl-3 with p50 or p52 homodimers is modulated by phosphorylation, which may differ with cells and conditions (Nolan et al., 1993; Caamaño et al., 1996). In any case, Bcl-3 is implicated in transactivation of κB-dependent reporters, differentiating this protein from other members of the IκB family. Despite these insights into possible mechanisms of action, the biologic contexts in which Bcl-3 functions are still unknown. Analyses of transgenic mice carrying a *bcl-3* transgene under the control of the lck promoter, which directs expression in thymocytes, also did not reveal a biologic function for Bcl-3 (Caamaño et al., 1996).

To gain physiologic insights into the role of Bcl-3, we generated Bcl-3 null mutant mice by targeted gene disruption. Such mice appeared to develop normally, but exhibited select defects upon immunologic challenge. Bcl-3-deficient animals dramatically failed in resisting infection with Toxoplasma gondii, an intracellular parasite, owing to lack of a protective T helper 1–like response. Bcl-3-deficient animals were impaired in germinal center formation and in formation of a proper microarchitecture in spleen and lymph nodes. These mice

[7]These authors made equally significant contributions and are listed in alphabetical order.

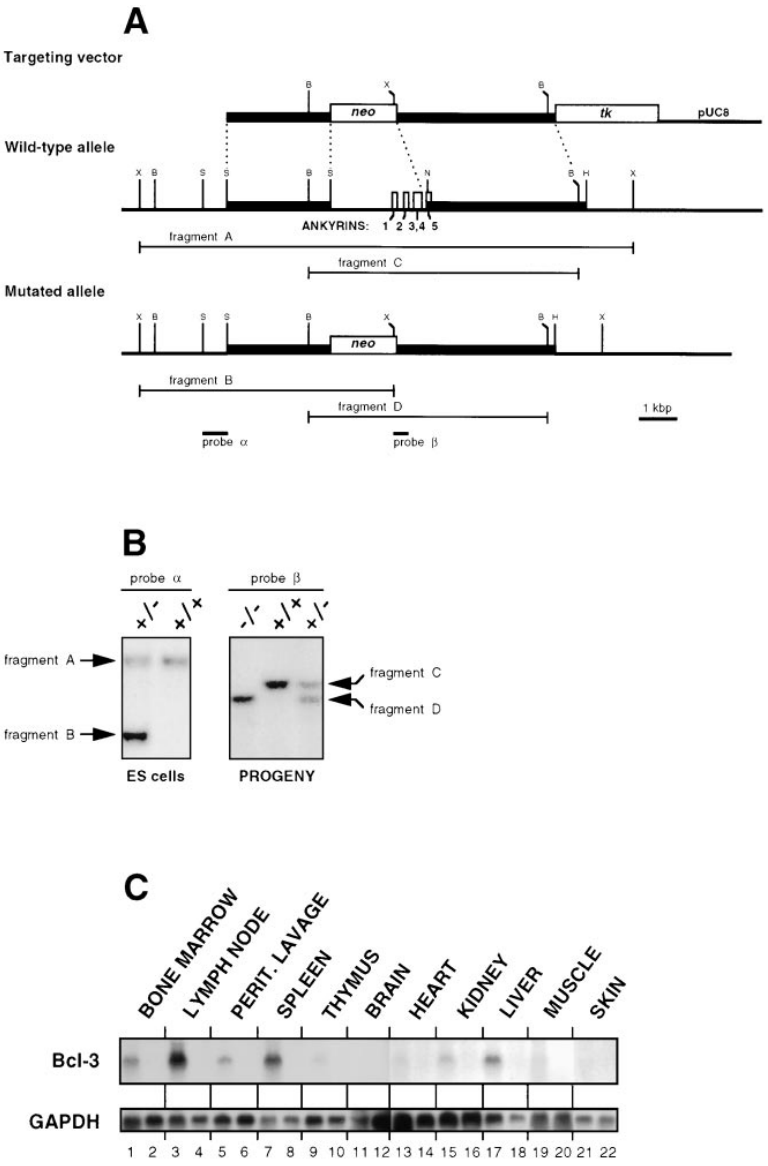

Figure 1. Targeted Disruption of the *bcl-3* Gene

(A) The targeting vector, partial exon–intron structure of the *bcl-3* gene locus, and mutated allele after homologous recombination of the targeting vector are shown. Probes used and fragments detected in Southern blots are indicated. Abbreviations are as follows: kbp, kilobase pairs; *neo*, neomycin; *tk*, thymidine kinase; *hsv*, herpes simplex virus; B, BamHI; S, SmaI; N, NotI; H, HindIII; X, XbaI.

(B) Southern blot analyses of ES cells (left panel) and of the offspring from the resulting mice heterozygous for the mutated allele (progeny; right panel). Correct homologous recombination in ES cells generates a new XbaI restriction fragment B (6.2 kb) in addition to the wild-type allele-derived fragment A (12.7 kb), and both were detected with probe α (a 0.45 kb SmaI fragment of BS-Not3). Correct insertion of the targeting vector was confirmed with additional analyses (data not shown). *bcl-3* +/−, +/+, and −/− mice in the offspring were identified with probe β, a 0.24 kb NotI–EcoRV fragment of *bcl-3* cDNA, which hybridizes to the BamHI fragments C (wild type) and D (mutant) (progeny panel).

(C) Northern blot analysis of *bcl-3* +/+ (odd lanes) and −/− mice (even lanes) for *bcl-3* mRNA expression (top panel; bottom panel shows expression of glyceraldehyde 3-phosphate dehydrogenase [GAPDH] as a control).

were also defective in proper antibody responses to influenza virus. Furthermore, a partial loss of B cells was noted. Bcl-3 may therefore contribute to the survival of B cells, which may relate to its oncogenic potential. The data suggest a role for Bcl-3 in select antigen-specific T and B cell responses and thus provide defined physiologic contexts in which this protein is critical.

Results

Targeted Disruption of the *bcl-3* Gene Locus

We generated mice lacking functional Bcl-3 by targeted disruption of the *bcl-3* locus in embryonic stem (ES) cells. Upon homologous recombination of the targeting construct, most of the ankyrin repeat–encoding exons of *bcl-3* were replaced by a neomycin cassette (Figure 1A), thus eliminating the domain responsible for physical interaction with NF-κB target complexes (see Introduction). Two out of six independently derived *bcl-3* mutant ES clones (B28 and B52) (analysis of clone B28 is shown in Figure 1B, left panel) were microinjected into C57Bl/6 blastocysts, and resulting male chimeras were bred to C57Bl/6 females to generate mice heterozygous for the mutated allele (via germline transmission). Matings of heterozygous animals, which displayed no obvious abnormalities, then generated *bcl-3* +/−, +/+, and −/− mice (Figure 1B, right panel). Both mutant ES cell lines allowed for germline transmission, and the two independently derived lines of mice were indistinguishable in all subsequent experiments.

As expected, Northern blot analysis of tissues obtained from *bcl-3* −/− mice failed to reveal any transcript when probed with a cDNA fragment encompassing the deleted exons of *bcl-3* (Figure 1C). In wild-type animals, *bcl-3* mRNA was detectable in most tissues at low levels,

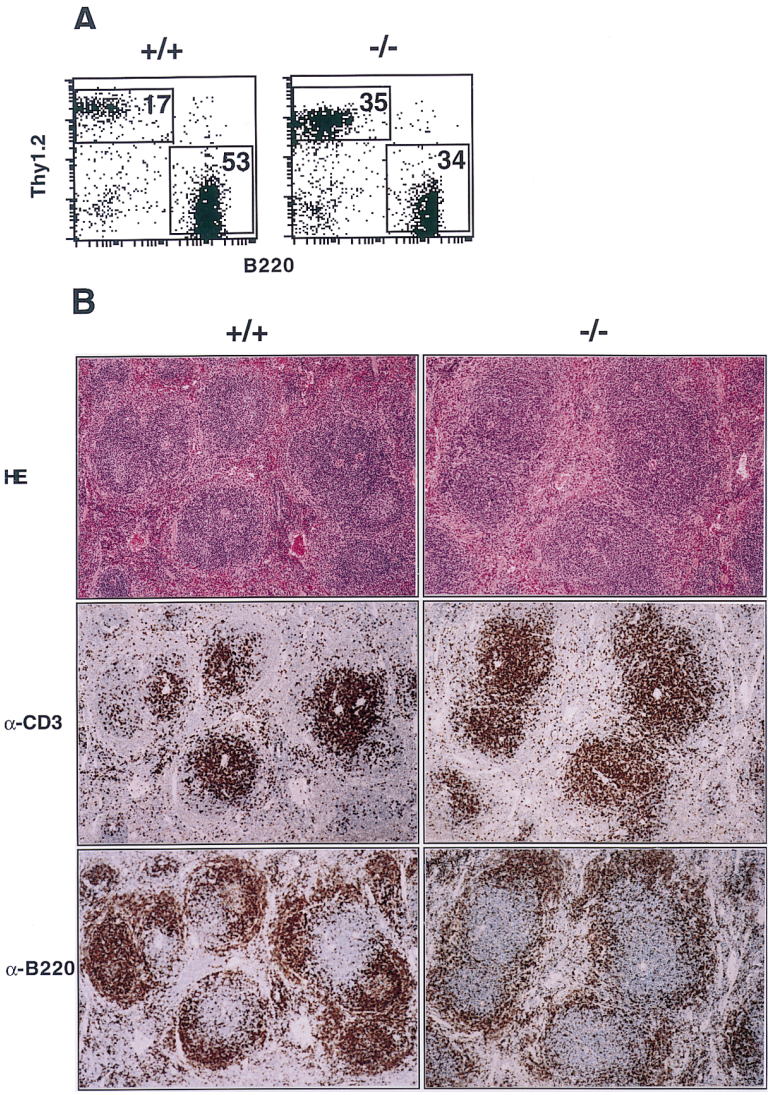

Figure 2. Absence of Germinal Centers and Reduction of the B Cell Compartment in *bcl-3* −/− Mice

(A) FCM analysis of splenocytes from 6-week-old *bcl-3* −/− and +/+ mice. Thy1.2–red 670 versus B220-PE two-color profiles are diplayed. Numbers in the quadrants reflect the percentage of total spleen cells in that quadrant.

(B) Absence of distinct B cell follicular organization and germinal centers in spleens of an unchallenged, 8.5-week-old *bcl-3* −/− mouse and a +/+ littermate control. Bouin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin (HE) or processed with anti-CD3 or anti-B220 antibodies, as indicated.

consistent with a prior report (Nolan et al., 1993). However, high levels of expression were quite restricted to certain tissues, in particular lymph nodes and spleen.

B Cell Population, Germinal Center Formation, and Antibody Responses

Homozygous *bcl-3* −/− mice were born with the expected frequency, developed normally, and showed no gross macroscopic abnormalities, except for variably hypoplastic lymph nodes (data not shown). Flow cytometric (FCM) analyses revealed a consistent reduction in the B:T cell ratio of Bcl-3-deficient animals in spleens (Figure 2A), as well as in blood and lymph nodes (data not shown). No significant additional abnormalities were apparent by FCM analyses in expression of the following surface markers in spleen, lymph nodes, bone marrow, and/or thymus of *bcl-3* −/− mice: CD3, CD4, CD8, αβTCR, Thy1.2, B220, CD28, CD44, HSA, Sca1, c-kit, CD25, CD69, NK1.1, GR-1, immunoglobulin D (IgD), IgM, CD5, CD45RB, I-A^b, and CD40 (data not shown).

The reduced B:T cell ratio in Bcl-3 null mice was confirmed by immunohistochemical analyses for B and T cells in paraffin-embedded tissue sections of spleen and lymph nodes. The B220-positive B cell compartment was reduced in size in unchallenged, Bcl-3-deficient animals as compared with their littermate controls. These mice largely lacked the well-organized, densely packed B cell follicles that were readily observed in spleens and lymph nodes of littermate controls (Figure 2B; spleen sections shown only; compare B220 +/+ with −/−). Thus, Bcl-3-deficient mice exhibited a partial loss of follicular B cells. Immunologic challenge with influenza virus or T. gondii led to further progressive loss of B cells from secondary lymphoid organs in mutant but not littermate control mice (data not shown; see below). It is possible that Bcl-3 has a role in maintaining the B cell population.

The stained sections also revealed the presence of some germinal centers within the B cell follicular areas of the littermate controls, which were most likely generated in response to environmental antigens (visible as

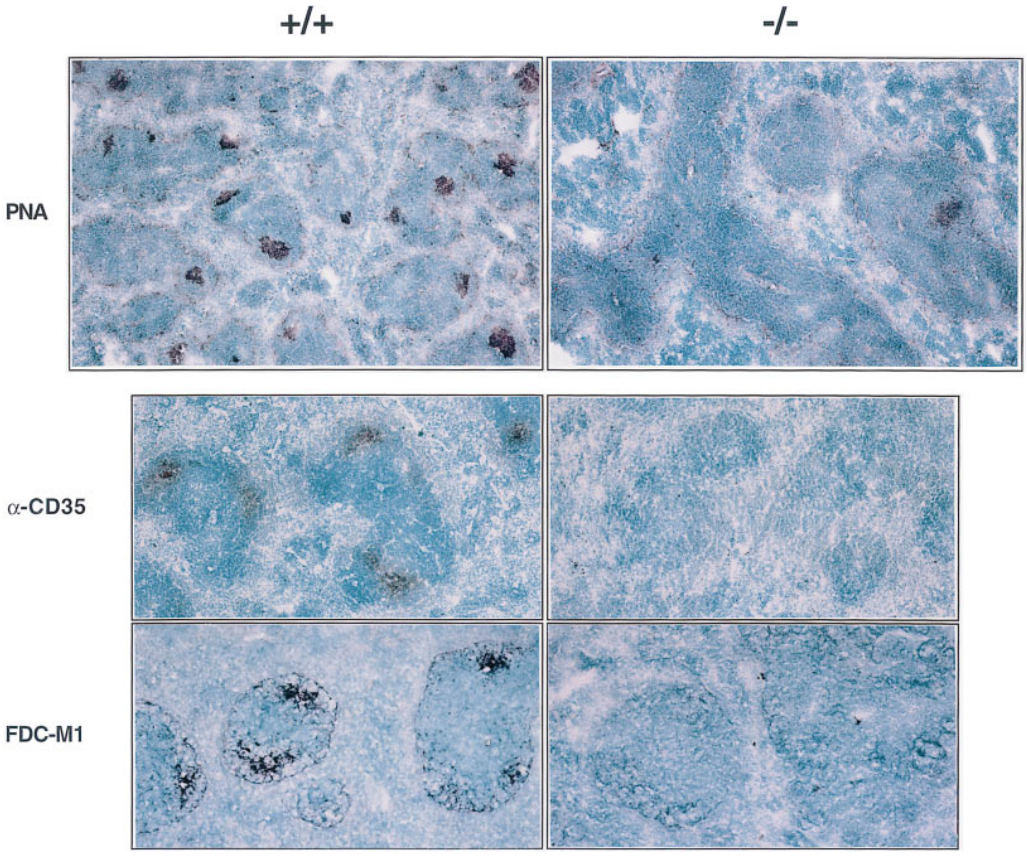

Figure 3. Impaired Formation of Germinal Centers in Spleens of *bcl-3* −/− Mice

We challenged 10- to 14-week-old *bcl-3* −/− mice and +/+ and +/− control mice (only +/+ is shown) by intraperitoneal injection of 100 μg of alum-adsorbed TNP-KLH. Frozen sections of spleens were obtained 8 or 17 days after injection or from uninjected animals and stained with anti-CD35 antibodies, PNA, or FDC-M1 antibodies, respectively. Stained cryosections from a representative pair of littermates are shown.

a lighter-staining center surrounded by a darker ring, which was evident with both hematoxylin and eosin and B-220; Figure 2B, +/+). Such centers were not noted in secondary lymphoid organs of Bcl-3-deficient animals (Figure 2B, −/−; only spleen shown), which may be related to the apparent impairment in B cell follicular structure. The inability of Bcl-3 null mutant mice to generate germinal centers was even more evident when mice were deliberately challenged with influenza virus or with the parasite T. gondii, since these centers were now frequently observed in littermate controls (data not shown).

While impaired in full germinal center reactions, Bcl-3-deficient mice may not be completely blocked in every aspect of these reactions, at least not when animals were injected with high amounts of TNP–keyhole limpet hemocyanin (TNP-KLH) antigen adsorbed to alum. Immunostaining of splenic cryosections revealed the presence of some peanut agglutinin (PNA)-staining cell clusters in challenged Bcl-3-deficient animals. PNA is considered a marker for germinal center B cells (MacLennan, 1994). Nevertheless, the numbers of these clusters were significantly lower than those seen in littermate controls (Figure 3; PNA; compare +/+ with −/− mice). Apart from the PNA marker, however, other indicators for germinal center reactions were completely lacking in the mutant mice, consistent with the data in Figure 2. Both CD35-positive cell clusters and follicular dendritic cell (FDC) clusters, which are normally associated with germinal centers (Schubart et al., 1996), could not be observed in Bcl-3 null mutant mice, but were readily detected in littermate controls (Figure 3; CD35 and FDCM1; compare +/+ with −/− mice).

Given the defect in germinal center formation, we tested the ability of *bcl-3* −/− animals to mount a humoral, T-dependent response to a physiologic challenge, infection with influenza virus. *bcl-3* −/− mice developed serum levels of Flu-specific IgM antibodies equivalent to levels measured in littermate control animals (Figure 4). The mutant mice did, however, exhibit a significantly impaired IgG2a antibody response, the dominant switched immunoglobulin isotype generated in response to influenza virus in wild-type mice (Coutelier et al., 1987) (Figure 4). The mutant mice did not generate any significant levels of other isotypes (data not shown). The remaining lower levels of Flu-specific IgG2a seen in some mice lacking Bcl-3 could be due to generation of some switched antibodies outside of germinal centers (see Discussion). Despite the impaired humoral response to influenza virus, serum titers for all

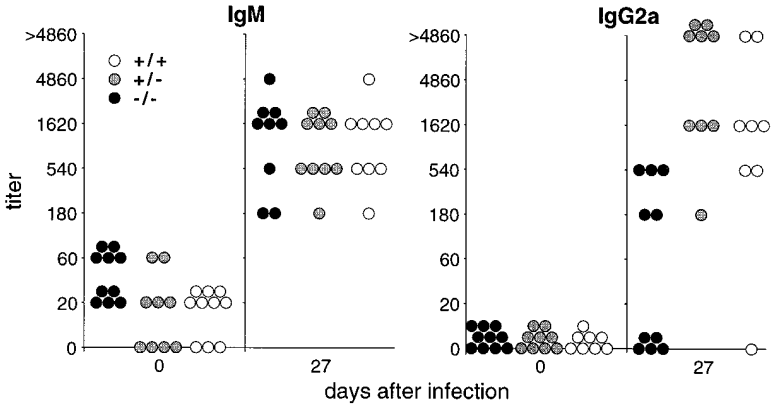

Figure 4. Defective Antibody Response in *bcl-3* −/− Mice

bcl-3 −/− mice produce reduced amounts of IgG2a (right panel) but normal amounts of IgM (left panel) antibodies after infection with influenza virus. Antibody titers prior to and 27 days after infection are shown for each genotype. Other influenza-specific antibody isotypes were at low levels even in control mice (data not shown). One of two independent experiments is shown. IgG2a anti-influenza titers differed significantly ($p < 0.001$) among the three groups (+/+, +/−, and −/−) by one-way analysis of variance. Pairwise comparisons identified the −/− mice as significantly different from each of the other groups, while +/+ mice did not differ from +/− mice.

immunoglobulin isotypes were within normal range in mutant mice (data not shown).

The Microarchitecture of Spleens

To probe for defects that may underlie impaired germinal center formation or antibody responses in Bcl-3-deficient animals, we further analyzed the splenic microarchitecture by immunostaining with various cell markers. While the B cell follicular zones appeared to be less well organized (see above), the T cell zones of spleen appeared grossly normal (see Figure 2B, CD3), including the presence of interdigitating dendritic cells (data not shown). The marginal zone (MZ) represents a third compartment of splenic white pulp, where distinct macrophage populations reside. In littermate control animals, marginal metallophilic macrophages (MMM) could be uniquely identified at the inner margin of the MZ with an anti-sialoadhesin receptor antibody (data not shown) or with the MOMA-1 antibody (Leenen et al., 1994) (Figure 5, +/+). In Bcl-3-deficient animals, in contrast, MMMs were dramatically reduced in numbers, and they no longer formed a continuous ring of cells (Figure 5, −/−; staining with anti-sialoadhesin antibodies gave similar results). Even more striking was the virtual absence of cells staining positive with ER-TR9 in mutant mice, while this antibody was clearly staining cells in littermate controls (Figure 5). ER-TR9 is the only unique antibody marker for marginal zone macrophages (MZM), which form a ring of cells in the outer portion of the MZ (Leenen et al., 1994). The finding suggests that MZMs

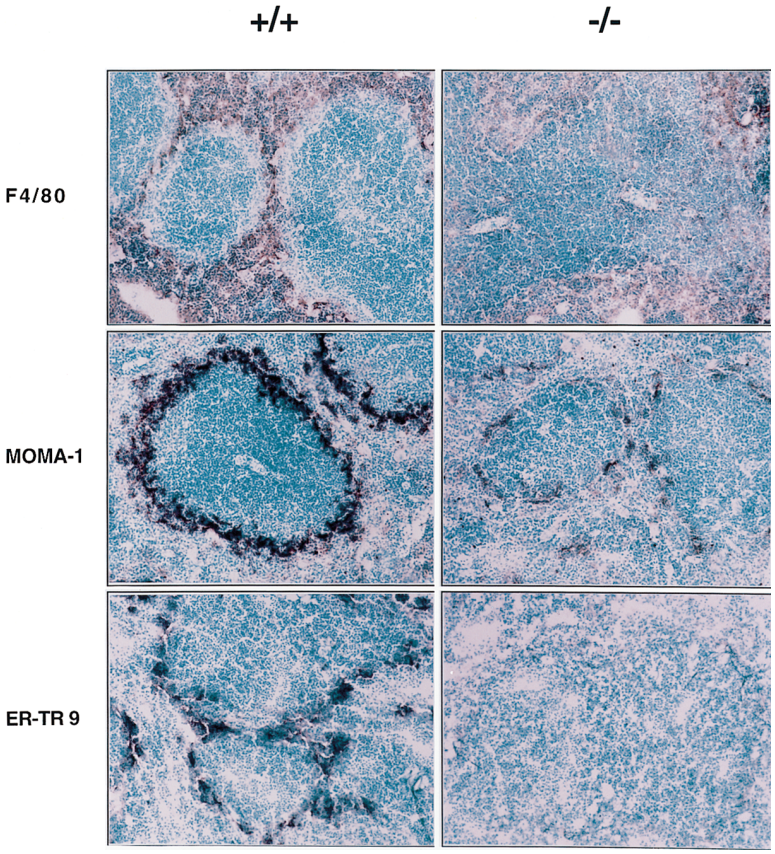

Figure 5. Altered Microarchitecture in Spleens of *bcl-3* −/− Mice

Splenic cryosections were derived from the same group of *bcl-3* −/− and +/+ mice that was used for Figure 3. Sections were stained with monoclonal antibodies specific to red pulp macrophages (RPM; F4/80), marginal metallophilic macrophages (MMM; MOMA-1), or marginal zone macrophages (MZM; ER TR-9), as indicated. Similar results were obtained with spleens from unchallenged animals (data not shown).

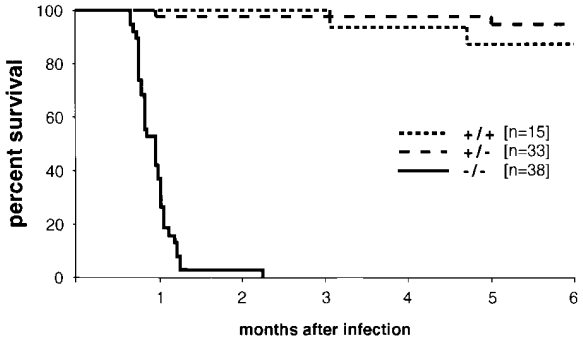

Figure 6. *bcl-3* −/− Mice Succumb to T. gondii Infection

bcl-3 −/− mice and +/− and +/+ littermates were injected intraperitoneally with approximately 20 cysts of the avirulent T. gondii strain ME49 (initially provided by Dr. J. Remington, Palo Alto Research Foundation), as detailed previously (Scharton-Kersten et al., 1996). The number of mice used for each group is indicated; 5- to 30-week-old mice of both sexes were used. Data are shown as percent survival calculated from three independent experiments. Both C57Bl/6 and 129SV genetic background strains are resistant to this parasite.

may be missing in MZs of Bcl-3-deficient animals, although a specific loss of the marker protein only cannot yet be ruled out. Taken together, the results indicate a general disruption of the MZ architecture in the mutant mice.

Finally, red pulp macrophages, which react with the MAC-1, F4/80, or BM-8 antibodies (Leenen et al., 1994), were readily detectable in both wild-type and mutant mice (Figure 5; only F4/80 is shown). However, while these cells were present exclusively in the red pulp of spleens from littermate control animals, they clearly infiltrated the white pulp of the Bcl-3-deficient spleens (F4/80, −/−; this was observed with the other markers as well; data not shown).

Infection with T. gondii

We investigated for potential defects in T cell–mediated immunity in a physiologically relevant infectious model. Mice were challenged with T. gondii, an intracellular protozoan that causes opportunistic infections in AIDS patients. In wild-type animals, parasites are initially controlled by the innate immune response of the host, thought to be mediated in large part by activated macrophages and natural killer (NK) cells (Gazzinelli et al., 1993). After about 1 week, an antigen-specific, T cell–mediated immunity begins to dominate, characterized by production of helper type 1 cytokines, and the infected animals progress from an acute phase to an asymptomatic chronic phase in which parasites are largely confined to dormant cysts (Gazzinelli et al., 1993). Control littermates (+/− and +/+) generally survived longer than 6 months after intraperitoneal injection of T. gondii. In contrast, Bcl-3-deficient mice failed to develop immunologic resistance and succumbed within a very narrow window of 3–5 weeks, with only 1 out of 38 infected mutant mice surviving slightly longer (Figure 6). Histopathologic examinations revealed that parasites became widely disseminated in Bcl-3-deficient animals during the course of infection, and this finding correlated with inflammation of multiple tissues (data not shown). Near time of death, the thymus became necrotic and secondary lymphoid organs nearly acellular; at this late point, T cells were also lost from secondary lymphoid organs, which occurred on top of the prior loss of B cells (data not shown; see above). The dramatic deaths of the null mutant mice, but not their littermate controls, documents a unique requirement for Bcl-3 in mounting a protective immune response to a natural pathogenic challenge; Bcl-3 was therefore essential for the survival of the host.

Interferon-γ (IFNγ) is a critical effector, helper type 1 cytokine for the protective immune response to T. gondii (Gazzinelli et al., 1993; Scharton-Kersten et al., 1996). During the first week of infection, soluble tachyzoite antigen (STAg) (Toxoplasma antigen preparation; Hakim et al., 1991) treated splenocytes from mutant mice and control littermates produced comparable amounts of IFNγ. Thereafter, however, the levels of this essential cytokine rapidly declined in Bcl-3-deficient animals, while littermate controls continued to produce high levels (Figure 7A). Initial parasite-induced production of IFNγ in wild-type mice is reported to derive primarily from activated NK cells, while at later times this cytokine derives from T. gondii–activated T cells (Gazzinelli et al., 1993; Hunter et al., 1994). Thus, the early rise in IFNγ in Bcl-3-deficient animals may be due to NK cells, while T cells may never produce this cytokine. Consistent with this notion, we observed that NK cytolytic activity, a general indicator of NK function, was normal in Bcl-3-deficient animals (Figure 7C), while T cell–specific production of IFNγ was defective throughout the entire course of infection. Purified, STAg-stimulated splenic T cells of infected, Bcl-3-deficient mice failed to produce IFNγ at all times, while the T cells of equivalently treated littermate controls could be shown to produce measurable amounts of this cytokine as early as 5 days after infection (Figure 7B). The T cell defect is present regardless of whether antigen-presenting cells were derived from wild-type or Bcl-3-deficient animals, although the defect was clearly more pronounced in the latter case. Defective IFNγ production by Bcl-3 defective T cells is not absolute, since stimulation with anti-CD3 antibodies led to normal induction of this cytokine (Figures 7A and 7B). Also, no absolute, cell-autonomous defects could be discerned in mitogenic responses of T cells from naive animals (data not shown). Therefore, in vivo antigen-dependent priming of T cells appeared to be defective in the mutant mice, while polyclonal stimulation in vitro was normal.

Nitric oxide (NO) directly kills parasites and is induced in macrophages, at least in part, by IFNγ (James, 1995). Consistent with the impaired production of IFNγ by T cells, we noted a significant reduction in the level of NO generated in response to STAg (Figure 7D). This may contribute to the failure to control the parasite. Furthermore, we detected impaired production of interleukin-12 (IL-12) by splenic cells of infected Bcl-3-deficient mice (Figure 7E), a cytokine essential for differentiation of T helper 1 cells (Gazzinelli et al., 1993; Trinchieri, 1995). While the defects in production of NO and IL-12 could be demonstrated ex vivo with cells derived from

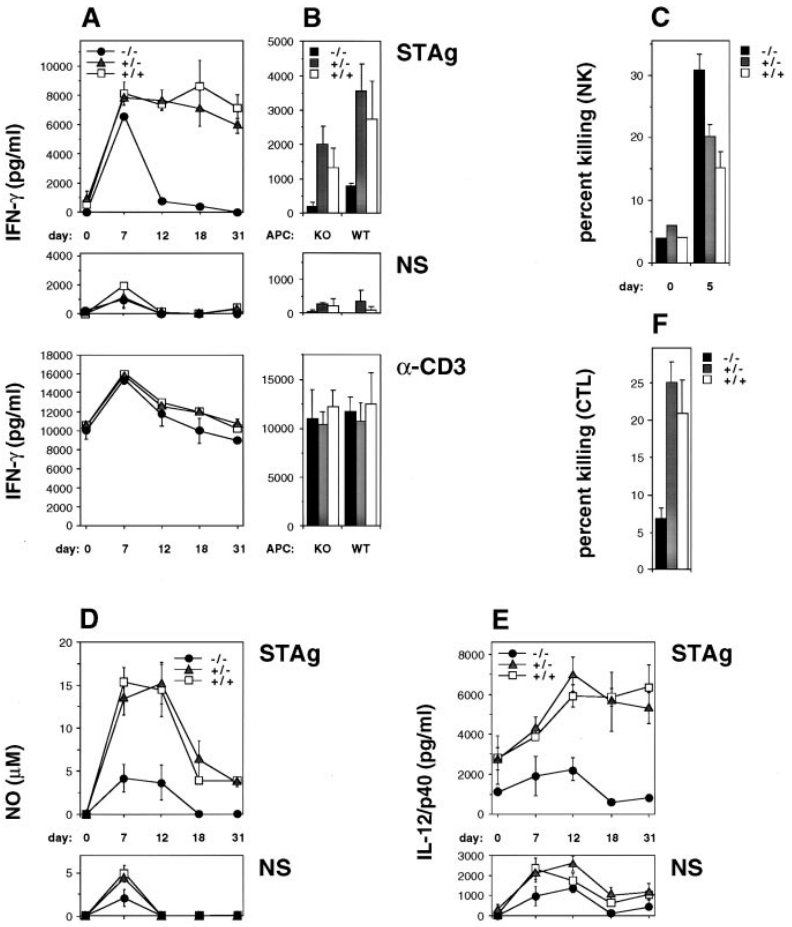

Figure 7. *bcl-3* −/− Mice Fail to Mount a T Helper Type 1–like Immune Response to T. gondii

(A) STAg-induced secretion of IFNγ by splenocytes isolated from *bcl-3* −/− mice at times postinfection as indicated (top panel). IFNγ production is impaired starting 7–12 days after infection with T. gondii. Also shown are results without stimulation (NS; middle panel) or with anti-CD3 stimulation (bottom panel).

(B) Splenic T cells purified from Bcl-3-deficient mice produce reduced amounts of IFNγ, detectable as early as day 5 after infection. Purity of cells (>97%) was assessed by FCM analysis using anti-CD3 antibodies (data not shown). Cells were mixed with antigen-presenting cells (APCs) isolated from infected *bcl-3* −/− mice (KO) or littermate controls (WT) and then treated with STAg (top panel) as in (A), cross-linked with anti-CD3 antibodies (bottom panel), or left untreated (NS; middle panel).

(C) NK cell cytotoxic activity is not impaired in *bcl-3* −/− mice. Spleen cells were collected from mice prior to or 5 days after infection, as indicated. Comparable results were obtained also with peritoneal exudate cells (data not shown). Results are expressed as percent killing obtained with a 50:1 effector to target ratio.

(D and E) Reduced production of nitric oxide (NO) and IL-12/p40 in response to STAg stimulation of splenocytes isolated from infected *bcl-3* −/− mice at times postinfection as indicated. Bottom panels show data without stimulation (NS) and top panels with STAg stimulation. Experiments in (A)–(E) were performed with mice infected in parallel with those yielding the survival curve of Figure 5, and mice were sacrificed at the indicated times (days after infection). The results are presented as the means (± standard deviations) of experimental data from at least three −/− mice, matched in each case with one or more littermate controls (+/− or +/+). Data shown were primarily from one set of infected mice. Two other sets of infected mice yielded essentially the same results. (F) CTL activity against T. gondii is impaired in *bcl-3* −/− mice. Data points were derived from analyses of four mice for each group. Effector to target cell ratios and calculations are as in (C).

Bcl-3-deficient, infected animals, these defects appeared not to be absolute in macrophages of naive animals, as judged by general stimulation of peritoneal macrophages in vitro. Thioglycollate-elicited macrophages of uninfected, Bcl-3-deficient mice responded normally with respect to all parameters measured, including induced production of NO and IL-12 as well as IL-6, tumor necrosis factor α (TNFα), IL-1β, H_2O_2, or surface expression of Fc receptors and class II antigens (data not shown).

The apparent defects in priming of antigen-specific T helper 1 cells may also be responsible for impaired cytotoxic T lymphocyte (CTL) activity against T. gondii–infected cells in Bcl-3 null animals (Figure 7F). CTL activity is an antigen-specific T cell effector function associated with host adaptive resistance (Hakim et al., 1991; Gazzinelli et al., 1993).

Discussion

We have generated mice which lack functional Bcl-3, and we have identified specific biologic defects in these mice. Bcl-3-deficient mice are impaired in select antigen-specific T and B cell responses, implying a requirement for Bcl-3 in these biologic processes in normal mice. Infection with the intracellular parasite T. gondii failed to generate antigen-specific, IFNγ-producing T cells in vivo. On the other hand, in vitro stimulation of T cells from Bcl-3 null mice resulted in normal IFNγ production, ruling out an absolute defect in induction of IFNγ. Instead, a critical defect during in vivo priming is indicated. The mutant mice were also unable to generate an appropriate IgG2a antibody response against influenza virus, while IgM responses were normal, as were circulating levels of all immunoglobulin isotypes. Consistent with this impaired antibody response to a T-dependent antigen, Bcl-3 null mice were essentially lacking in germinal center formation, an important anatomical site for the generation of high affinity, isotype-switched antibodies against T-dependent antigens. A disruption of the splenic architecture, and in particular the partial or complete loss of macrophage populations resident in the splenic MZ, could underlie observed defects in antigen-specific priming events in vivo. Finally, impaired antigen-driven cellular interactions may also

be involved in the progressive loss of B cells in Bcl-3-deficient animals. Bcl-3 may normally function as an integral regulator of antigen-dependent cellular signaling cascades and, as such, may play a role in the survival of B cells, consistent with its tumorigenic potential.

Impaired Adaptive Immunity to T. gondii

Infection of Bcl-3-deficient animals with T. gondii resulted in death of the animals within 3–5 weeks, while wild-type or heterozygous littermates survived for longer than 6 months, with few exceptions. This result clearly emphasizes the important physiologic role Bcl-3 plays in fighting this pathogen. Extensive analyses of the responses during the course of infection with T. gondii indicated that the mice specifically failed to establish a proper antigen-specific T helper 1 response. Production of both IL-12 and IFNγ were impaired, two cytokines necessary for generation of a normal T helper 1 response (Biron and Gazzinelli, 1995; Trinchieri, 1995). IFNγ is also essential for elimination of the parasites (Gazzinelli et al., 1993), a fact which is underscored by the rapid death, at about 9 days, of T. gondii–infected mice rendered null for this cytokine (Scharton-Kersten et al., 1996). Bcl-3-deficient animals could be shown to produce some IFNγ from sources other than T cells (most likely NK cells) for up to 1–2 weeks postinfection, but not thereafter. An intact early (but transient) innate protection mechanism also explains why Bcl-3-deficient animals survived for as long as 3–5 weeks, past the initial acute phase of infection. In contrast, the T cell–mediated, antigen-specific helper type 1 response was clearly defective, and animals succumbed at a time when resistance depended on antigen-specific T cell functions. It remains to be determined whether T helper 2 responses or T helper 1 responses to other pathogens are impaired as well.

Defects in Germinal Center Formation and Antibody Responses

The antibody response to influenza virus was defective. Levels of anti-Flu IgG2a, the dominant switched immunoglobulin isotype to this antigen, were significantly reduced. The antibody defect correlated with impaired formation of germinal centers in infected Bcl-3 null mice. In contrast, germinal centers were readily formed in the B cell follicles of littermate controls. Such centers are the primary anatomical sites where antigen-specific B cells undergo rapid expansion, coupled with somatic hypermutations of variable region genes and isotype switching, followed by selection based on affinity to antigen, and, finally, differentiation into plasma cells or memory cells (MacLennan, 1994; Kelsoe, 1995). Much of this biologic program occurs in close proximity with antigen-specific T helper cells and clustered antigen-presenting follicular dendritic cells (FDCs) (Tew et al., 1993; Grouard et al., 1995; Kosco-Vilbois and Scheidegger, 1995).

However, Bcl-3-deficient animals were not always completely blocked in generation of switched antibodies to T-dependent antigens, nor did they appear to be completely blocked in every phase of germinal center–like reactions. When challenged with high dose TNP-KLH adsorbed to alum, these mice generated an apparently normal antibody response (unpublished data). In addition, a few PNA-positive B cell clusters were observed in spleen under these conditions. Nevertheless, neither CD35-positive cell clusters nor FDC clusters were present, confirming the absence of proper germinal centers. In any event, recent evidence indicates that antibody responses to T-dependent antigens do not strictly depend on germinal center reactions and may occur without them, since lymphotoxin α (LTα)-deficient mice challenged with high dose (but not low dose) T-dependent antigen generated normal antibody responses, despite a complete absence of germinal centers (Matsumoto et al., 1996a).

Disruption of the Splenic Architecture: The MZ

Immunohistochemical analyses of spleen revealed disruptions of the microenvironment, in particular the MZ, which could underlie at least some of the defects seen in antigen-dependent T and B cell responses in vivo. The MZ is a distinct microanatomical site which surrounds the white pulp areas and represents the border with the red pulp. It is of special interest since this zone is first to encounter and trap blood-borne antigens and potentially display them for presentation to lymphocytes of the white pulp. The MZ also regulates cellular trafficking into the white pulp, consistent with its strategic border location (Liu and Banchereau, 1996). MZs contain two distinct macrophage populations, marginal metallophilic macrophages (MMM), located on the inner portion of the MZ, and marginal zone macrophages (MZM), located on the outer portion (Leenen et al., 1994). In Bcl-3-deficient animals, a marker for MZMs could no longer be detected and MMMs were dramatically reduced in frequency and formed a disturbed pattern. Thus, a disruption of the MZ architecture was in evidence, although markers for MZ endothelial cells (Briskin et al., 1993) and reticular fibroblasts (Van Vliet et al., 1986) were still detected (unpublished data). The inferred functional defects of this border between red and white pulp are supported by the abnormal entry into the white pulp of macrophages, which normally reside in the red pulp only. Taken together, the data imply defects in cellular trafficking and possible antigen-presenting functions, defects which may interfere with select critical cellular interactions necessary for antigen-driven T helper 1 responses to T. gondii and germinal center reactions or antibody responses.

Mice with Related but Distinct Phenotypes

Disruption of secondary lyphoid microarchitecture or impaired germinal center reaction (or both) has been observed in several other mice, including mice deficient in LTα (DeTogni et al., 1994; Matsumoto et al., 1996b; see also Ettinger et al., 1996), TNFα (Pasparakis et al., 1996), or TNF receptor 1 (LeHir et al., 1996). Loss of germinal centers has been noted in mice lacking the membrane proteins CD40, CD40 ligand (Kawabe et al., 1994; Xu et al., 1994; Grewal et al., 1995), CD28 (Lane et al., 1994; Ferguson et al., 1996), major histocompatibility

complex class II (Cosgrove et al., 1991), CD19 (Rickert et al., 1995), or the complement receptors CR1/CR2 (Ahearn et al., 1996); furthermore, the intracellular tyrosine kinase Lyn (Hibbs et al., 1995; Nishizumi et al., 1995) and OBF-1/OCA-B/Bob1, the B cell–specific coactivator of Oct-1 and Oct-2 (Kim et al., 1996; Schubart et al., 1996). Of note, CD40- and CD40 ligand–deficient mice share several phenotypes with Bcl-3-deficient mice: not only are they impaired in germinal center formation and unable to generate antibodies with switched isotypes to T-dependent antigens, but they also fail to establish a T helper 1 phenotype when challenged with leishmania (Campbell et al., 1996; Kamanaka et al., 1996; Soong et al., 1996). Despite this wealth of information, specific molecular mechanisms that underlie the observed defects in these mutant mice remain unknown.

Mice deficient in the NF-κB proteins p50/p105 (Sha et al., 1995; Michaelson et al., 1996; Snapper et al., 1996), RelB (Burkly et al., 1995; Weih et al., 1995), c-Rel (Kontgen et al., 1995; Gerondakis et al., 1996), and p65 (Beg et al., 1995a) and in the IκB-α protein (Beg et al., 1995b; Klement et al., 1996) have been reported. However, none appear to have phenotypes that resemble those described here for Bcl-3 null mutants. The results obtained with these and our mice do suggest that the regulatory potential of the NF-κB/IκB system is most fully utilized by the immune system.

Future Considerations

The cells and the gene products most critically affected by the absence of Bcl-3 remain to be determined. The presumably complex cellular interactions that are required for splenic microarchitecture, germinal center reactions, and/or antigen-specific priming are not understood, and thus the specific role(s) of Bcl-3 cannot yet be anticipated. It is also unknown whether Bcl-3 is functionally related to the proteins discussed above that have apparently critical roles in these biologic processes. It is possible that Bcl-3 carries out important functions in select cells such as MZMs or that it is essential in select signaling paths, which may not be readily analyzed in vitro. For example, FCM analyses of splenocytes for expression of CD40 and inducible CD40 ligand on T cells (and B7-2 on other cells) appeared grossly normal, as did proliferation of purified B cells mediated through signaling via CD40 or IgM (or both), although the response was somewhat blunted in the latter case (unpublished data). In any event, such in vitro experiments cannot rule out subtle yet potentially significant defects associated with these or other signaling proteins in Bcl-3-deficient animals.

The function(s) Bcl-3 executes in building or maintaining the microarchitecture of secondary lymphoid organs may be required for at least some T helper 1 responses and some T-dependent antibody responses. Furthermore, survival of B cells may depend on these same or similar functions. In the absence of Bcl-3, B cells appeared less well organized in secondary lymphoid organs and their numbers were reduced. The implied role of Bcl-3 in B cell survival may explain its oncogenic activity when expressed at high, constitutive levels as a result of chromosomal translocation. Preliminary analyses of p52/p100-deficient animals generated in our laboratory so far suggest that a number of phenotypes (but not all) are shared with Bcl-3 null mice. In contrast, the phenotypes of p50/p105-deficient mice appear distinct, including their ability to form germinal centers (unpublished data). This lends support to the notion that a physiologically relevant complex of p52 and Bcl-3 exists in vivo, as predicted by previous work in vitro (see Introduction). The work presented establishes specific and physiologically important contexts in which Bcl-3 is required in vivo, which provides a basis to explore these biologic processes further, elucidate underlying molecular mechanisms, and define the role of Bcl-3 in tumorigenesis.

Experimental Procedures

Targeted Disruption of the *bcl-3* Locus

A genomic phage clone carrying ankyrin repeat sequences 1–6 of Bcl-3 was isolated from a 129SV mouse genomic library (Stratagene). Two adjoining genomic NotI fragments, Not3 (10.4 kb) and Not2A (7.2 kb), were obtained from this phage and were cloned into Bluescript SK(−) (BS SK−; Stratagene), partially sequenced, and mapped for restriction sites (see Figure 1A). To construct the targeting vector, a 4.3 kb NotI–HindIII fragment of BS-Not2A, which carries exons encoding ankyrin 6 and part of ankyrin 5, was inserted into the BamHI site of the pPNT vector (Tybulewicz et al., 1991), immediately 3′ to the *neo* gene and 5′ to the *hsv-tk* gene. Subsequently, the 2.85 kb SmaI fragment derived from BS-Not3 was inserted into the XhoI site (just 5′ to the *neo* gene) after filling in by Klenow reaction to generate pPNT-BCL3KO. 50 μg of the targeting construct (pPNT-BCL3KO) were linearized with NotI and electroporated into 10^7 J1 ES cells (passage 12), as detailed elsewhere (Cao et al., 1995). Positive-negative selection was started 24 hr after transfection using G418 (GIBCO BRL; 350 μg/ml) and ganciclovir (2 μM). Screening of 75 G418/ganciclovir-resistant clones revealed six correctly recombined clones (see Figures 1A and 1B for more details).

FCM Analyses

Three-color flow cytometric (FCM) analyses of single cell suspensions of spleens were performed with anti-B220-phycoerythrin (PE)-, anti–CD3-fluorescein isothiocyanate (FITC)- (data not shown), and anti-Thy1.2-biotin-conjugated antibodies (Pharmingen), followed by streptavidin–red 670 (GIBCO BRL), as previously described (Shores et al., 1994).

Infection with Influenza Virus and Isotyping of Antigen-Specific Antibodies

We challenged 6- to 19-week-old mice via intranasal injection under metophane anesthesia of 50 μl of a PBS suspension of the A/Philippines influenza strain (250 tissue culture infectious $dose_{50}$ [$TCID_{50}$]). Mice were rechallenged 22 days later by intraperitoneal injection of 1 ml of PBS containing approximately 200 hemagglutinating units (HAU) of the A/PR8 strain. Bleeds were collected on day 27 after initial infection, and preimmune sera were harvested 24 hr before primary challenge. Titer of antibodies to influenza virus were determined by isotype-specific enzyme-linked immunosorbent assay (ELISA). We coated 96-well plates (Nunc) overnight at 4°C with formalin-inactivated influenza vaccine, A/Philippines. After blocking (1% fetal calf serum, 0.05% sodium azide in PBS) and between subsequent steps, plates were washed four times with PBS-T (0.05% Tween 20, 0.05% sodium azide in PBS). Serial dilutions of sera were then transferred onto virus-coated plates and incubated for 2 hr. Alkaline phosphatase–conjugated goat antibodies to various mouse immunoglobulin isotypes (Southern Biotechnology Associates) were added to washed plates and incubated for 1 hr. Plates were finally washed and substrate was added (1 mg/ml p-nitrophenyl phosphate in 1 mM $MgCl_2$, 50 mM Na-bicarbonate buffer [pH 9.8]). Optical density was measured with a Titertek MultiScan ELISA plate reader at 414 nm.

Immunoperoxidase Staining of Paraffin-Embedded and Frozen Tissue Sections

Lymph nodes and spleens were fixed in Bouin's fixative for 24 hr, rinsed, and transferred into 70% ethanol. Tissues were then processed through alcohols and xylene, embedded in paraffin, sectioned at 5 μm and either stained with hematoxylin and eosin or used for immunohistochemistry. Sections were stained with an anti-human CD3 rabbit polyclonal antibody (Dako Corporation) or an anti-mouse B220/CD45R biotin-conjugated rat monoclonal antibody (Boehringer-Mannheim). An anti-rabbit biotin-conjugated secondary antibody was subsequently used for the CD3 stain (Vector Laboratories). Slides were then incubated with streptavidin-conjugated horseradish peroxidase (HRP), and the avidin–biotin complexes were revealed with 3,3′-diaminobenzidine (DAB) tetrahydrochloride chromogen (Sigma).

For frozen sections, spleens were extracted, placed in OCT freezing medium (Miles Laboratories, Inc.), and flash frozen. Acetone-fixed sections (10 μm) were stained as described previously (Kelsall and Strober, 1996). In brief, tissue sections were rehydrated in phosphate-buffered saline (PBS) containing 0.1% BSA (fraction V, PBS/BSA; Sigma) and then blocked for 30 min with 20% normal mouse serum and 20% goat or rabbit serum (same species as secondary antibody) in PBS (PBS/serum). After blocking, sections were incubated for 60 min with the primary antibody prepared in PBS/serum, washed in PBS/BSA, and then incubated for an additional 30 min with the biotinylated secondary antibody in PBS/serum (except for those stained with biotinylated PNA or biotinylated anti-CD35 antibodies). After quenching endogenous peroxidase activity, tissue sections were incubated for 30 min with streptavidin-conjugated HRP (Vector Laboratories) and washed in PBS/BSA, and the avidin–biotin complexes were revealed with the DAB chromogen (Vector Laboratories), according to the instructions of the manufacturer. Finally, slides were rinsed, counterstained with methyl green (Vector Laboratories), and permanently mounted with Permount (Fisher Scientific). Sources of reagents were as follows: biotinylated PNA (Pierce); FDC-M1 (anti-FDCs; a gift of Drs. M. H. Kosco-Vilbois and G. Burton); biotinylated anti-CD35 (Pharmingen); F4/80 (anti–red pulp macrophage; Caltag Laboratories); MOMA-1 (anti-marginal metallophilic macrophages; BACHEM Bioscience Inc.); ER TR-9 (anti-marginal zone macrophages; BACHEM Bioscience Inc.).

Cell Culture, Stimulations, and Cytokine Measurements

Single cell suspensions were prepared from spleens and, after lysing of erythrocytes, cells were washed and cultured at 37°C in RPMI medium (Scharton-Kersten et al., 1996). For Figures 7A, 7D, and 7E, cells were cultured in 24-well plates (5×10^6 cells per well) in a total volume of 1 ml and treated with STAg (5 μg/ml) (Hakim et al., 1991) or cross-linked anti-CD3ε antibodies (145-2C11) (Pharmingen), as indicated. For the stimulation with anti-CD3 antibodies, plates were coated overnight at 4°C with a 50 μg/ml solution of 145-2C11 antibody in PBS and then washed three times before addition of the cell suspensions. Concentrations of IL-12/p40, NO, and IFNγ were assayed on supernatant aliquots 24, 48, or 72 hr later, respectively. IFNγ was assayed by two-site ELISA, as previously described (Scharton-Kersten et al., 1996). Nitrite levels, reflecting the NO levels in samples, were measured by the Griess reaction (Scharton-Kersten et al., 1996). IL-12/p40 was assayed by two-site ELISA, as previously described (Scharton-Kersten et al., 1996), using monoclonal antibodies derived from cell lines originally generated by M. Wysocka and G. Trinchieri (Vieira et al., 1994). Recombinant IL-12 (Genetics Institute) was used as a standard in the assays. For experiments shown in Figure 7B, T cells were purified by negative selection from spleens of *bcl-3* −/−, +/−, and +/+ mice, which had been infected with T. gondii 5 days earlier. We preincubated 10^8 splenocytes with 10 μg of each of the following antibodies: anti-NK-1.1 (Pharmingen), anti-Mac-1 (Boehringer-Mannheim), anti-CD11c (Endogen), anti-I-A^b (clone M5/114 from Boehringer-Mannheim and clones AF6-120.1, KH74, and 25-9-3 from Pharmingen), and anti-CD45R/B220 (Pharmingen). Cells were then washed, further incubated with magnetic beads coated with goat anti–mouse IgM and IgG and anti–rat IgG (PerSeptive Diagnostics) and passed over a magnet. Antigen-presenting cells were obtained by complement-mediated T cell depletion of spleen cell suspensions and γ-irradiation (3300 rads) according to standard procedures. We cocultured 1.5×10^6 T cells from individual mice in 24-well plates in 800 μl of medium with 5×10^6 antigen-presenting cells pooled from spleens of *bcl-3* −/− (knockout) or +/+ and +/− (wild type) mice, as indicated. Cells were treated as specified and IFNγ was measured 72 hr later, as described above.

Cytotoxicity Assays

NK cell–mediated lysing of target cells was carried out as described (Scharton-Kersten et al., 1996) with effector spleen cells obtained from mice prior to or 5 days after infection with T. gondii. For the CTL assays, mice were immunized by intraperitoneal inoculation of 2×10^4 live tachyzoites of the ts-4 strain of T. gondii (Hakim et al., 1991) and boosted after 15 and 30 days with the same dose. CTL activity against T. gondii was measured in single spleen cell suspensions 6 weeks after the last boost, as detailed elsewhere (Hakim et al., 1991). In brief, responder cells (5×10^6) from vaccinated mice were cultured in 2 ml in 24-well plates with 10^6 γ-irradiated (15,000 rads) ts-4 tachyzoites for 5 days. The presence of a similar percentage of $CD8^+$ cells in the different cultures was assessed by FCM analyses performed just before the assay. Resident peritoneal macrophages from C57Bl/6 mice were infected with the RH strain of T. gondii and used as target cells. Uninfected macrophages were also used to calculate the specific ^{51}Cr release. For both NK and CTL activity, effector to target ratios were 100:1, 50:1, 25:1, and 12:1. The specific ^{51}Cr release into supernatants (percent killing) was calculated from the effector:target ratio of 50:1 as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100%. Maximum and spontaneous ^{51}Cr release values were calculated by adding 10% sodium dodecyl sulfate (SDS; Sigma) or medium alone, respectively, in place of effector cells.

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